

Polybrominated Biphenyls in Model and Environmentally Contaminated Human Blood: Protein Binding and Immunotoxicological Studies

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A review and summary is given of analytical, biochemical, and immunological studies made following an immunodiagnostic investigation which revealed significant decreases in the numbers, and changes in the functional integrity, of both T- and B-lymphocytes in a group of Michigan dairy farmers exposed to polybrominated biphenyls (PBB) which had been inadvertently introduced into the food chain in 1973. A quantification technique based on selected ion monitoring of bromine anions, obtained in negative chemical ionization, permitted determination of 10–35 pg of individual PBB congener per mL serum, a 20-fold improvement over electron capture gas chromatography. An *in vitro* spiked system was established and shown to be a representative model of environmentally contaminated blood. Immunoprecipitation followed by mass spectrometric quantification determined that the distribution of PBB among plasma, erythrocytes, mononucleocytes and polymorphonucleocytes was 89:9:<1:<1. In plasma 80% of the PBB was bound to apolipoproteins B and A in a 3:1 ratio. No preferential absorption of PBB congeners was found in the blood compartments suggesting that changes in the relative abundances of PBB congeners observed in longitudinal studies on Michigan subjects reflect differences in excretion rates or metabolism. A repeat in 1981 of the immunodiagnostic tests conducted in 1976 revealed a virtually complete persistence of the immune dysfunctions in the Michigan farmers exposed to PBB a decade ago.

Introduction

In 1973, livestock feed for dairy cattle in Michigan was contaminated with polybrominated biphenyls (PBB) when Firemaster BP-6, an industrial fire retardant, was mistaken for Nutrimaster, a magnesium oxide cattle feed supplement. The resulting toxicosis in animals led to the quarantining of 500 farms and the destroying of 32,000 cattle, 1.6 million chickens, and 5 million eggs. Acute PBB-related syndromes were reported among chemical workers directly exposed to PBB and farmers who commonly consumed their own dairy and meat products. As a result of the statewide distribution of dairy produce and meat from the contaminated farms, the majority of Michigan residents were exposed to PBB during the 5 years after the contamination.

The recognition of the potentially serious long-term consequences of this accident prompted extensive animal and *in vitro* studies on the hepatotoxic, neurotoxic, immunotoxic, and carcinogenic properties of PBB, and the establishment of cohorts of thousands of exposed

persons to serially monitor blood levels in 1–2 yr intervals. Such studies have been continuing to date, even though the contamination ended in 1978, and the manufacture of PBB was banned in 1978. A recent review summarizes the scientific as well as the political aspects of the PBB contamination in Michigan and provides a list of relevant references (1). It has been estimated that 90% of the population of the State of Michigan now carries a body burden of PBB (2). Continued interest in PBB is justified by the potential of long-term and as yet undetected medical consequences, e.g., decreased developmental ability of children after neonatal exposure to PBB (3) and of PBB uptake due to the extremely slow excretion/metabolism of this class of compounds (4,5).

Since 1976, the Environmental Sciences Laboratory and the Immunotoxicology and Analytical Biochemistry Laboratories of the Department of Neoplastic Diseases of the Mount Sinai School of Medicine have been conducting epidemiological, comprehensive clinical and neurological, immunotoxicological, analytical, and biochemical studies on Michigan farm residents, Michigan chemical workers, and controls from Wisconsin farms and New York City. The present paper reviews the work

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done in our laboratories on the analytical chemistry, protein binding, and immunotoxicological effects of PBB; also included are results of pilot experiments on alternative approaches to the study of protein binding, and some longitudinal data on immunoglobulins.

Quantification by Selected Ion Monitoring

Gas chromatography with electron capture detection (EC-GC) has been the primary analytical technique for the quantification of PBB in body fluids and fatty tissues in body burden studies (6-8). The concentration of PBB in serum is usually expressed in terms of the most abundant hexa isomer (2,2',4,4',5,5'-hexabromobiphenyl, HxBB), and the detection limit for HxBB in serum by EC-GC is approximately 1 ng/mL (1 pbb); blood levels of this order have been reported in many Michigan subjects (9).

The possibility that PBB congeners are preferentially accumulated or excreted has been suggested in studies in Michigan farmers and chemical workers (10,11) as well as in experimental animals (12), and attention was called to the potential importance of several minor components of Firemaster (13). The positive identification and quantification of PBB congeners other than the most abundant HxBB in the general population requires analytical techniques which are more specific and have lower limits of detection than EC-GC.

A pilot study on the use of positive chemical ionization mass spectrometry with selected ion monitoring (14) revealed that a vast improvement in specificity can be provided when the protonated molecular ions of individual PBB are monitored. Detection limits, however, appeared inadequate for the determination of blood levels in the general population. A subsequent investigation of the mechanism of halogen anion formation by polybrominated compounds in negative chemical ionization mass spectrometry revealed that dissociative electron capture by polybrominated compounds leads to the formation of bromine anions in great abundance (15). This observation led to the development of a complete analytical methodology for the quantification of individual PBB in serum by the selected ion monitoring of bromine anions ($m/z = 79$) obtained in negative chemical ionization with nitrogen as the reagent gas (16). Detection limits were 10 pg/mL for pentabromobiphenyls and 35 pg/mL for the other PBB congeners, an approximately 20-fold improvement over electron capture gas chromatography. Although this technique is not as specific as the one based upon the monitoring of protonated molecular ions in positive chemical ionization or the molecular anions in negative chemical ionization, specificity is still much higher than in EC-GC because only bromine yielding compounds, with GC retention times identical to those of PBB (a highly unlikely occurrence), may conceivably interfere (particularly when the $m/z = 81$ peak is comonitored).

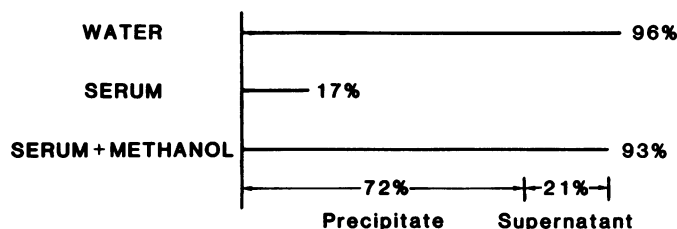


FIGURE 1. Recovery of added ^{14}C -2,2',4,4',5,5'-hexabromobiphenyl from water and serum under various sample preparation conditions (see text).

Regardless of the analytical technique used for detection and quantification, the extraction of PBB from the serum (plasma) samples must be as complete as possible. Figure 1 shows the recovery of PBB by extraction (twice) with 5-fold volumes of hexane-diethyl ether (1:1, v/v) under various conditions. These experiments were carried out using universally labeled ^{14}C -HxBB (specific activity: 10.2 mCi/mmol). The top line of Figure 1 shows that 96% recovery can be achieved from water. The middle line shows that only 17% of HxBB can be recovered from serum with a straight extraction with hexane-diethyl ether. The strength of the binding of PBB to serum proteins, which is sufficient to prevent efficient solvent extraction, can be reduced significantly by adding 2 volumes of methanol (and vortexing) prior to extraction. It is seen that 93% recovery is possible when serum is first treated with methanol to precipitate proteins and the precipitate is not removed prior to extraction with hexane-diethyl ether. PBB is still bound to protein after the methanol treatment but the bonds are apparently weakened to such a degree that the addition of the solvent mixture can completely release all PBB present. When the methanol precipitate is removed by centrifugation prior to extraction, and the precipitate and supernatant are extracted separately, the amounts extracted are 72% and 21%, respectively (Fig. 1). The lipophilic nature of PBB suggests that the nonprotein-bound fraction is associated with the free lipids of serum.

Protein Binding

Binding to Individual Serum Proteins

Figure 2 summarizes the results of a series of experiments where ^{14}C -HxBB (20 ng) was incubated (37°C, 1 hr) separately with γ -globulin, albumin, and apolipoprotein B (2-40 mg each) dissolved in water. It is seen that even in a noncompetitive situation the PBB is bound most efficiently by proteins with hydrophobic components.

In Vitro Models

The protein binding behavior of PBB in environmentally contaminated blood has been successfully mimicked by spiking whole blood as well as plasma/serum with either ^{14}C -HxBB or individual congeners (incuba-

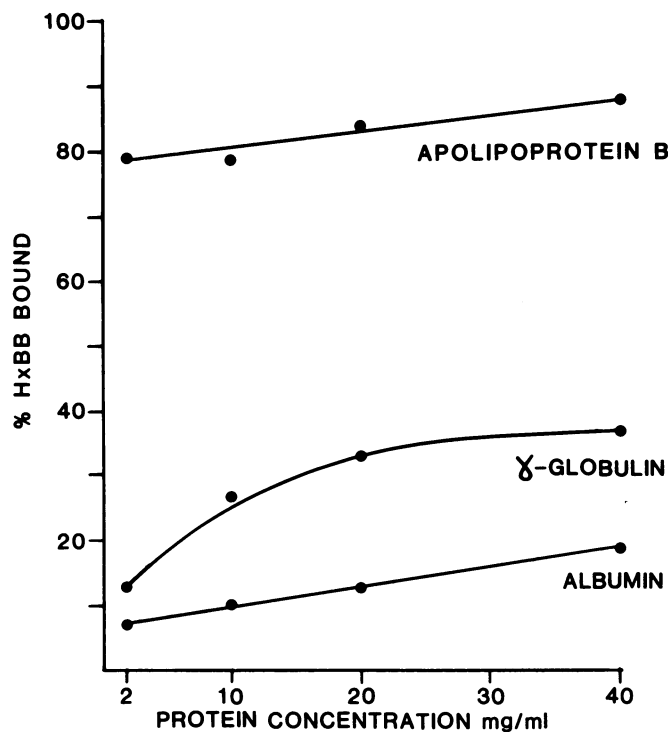


FIGURE 2. Percentage of ^{14}C -2,2',4,4',5,5'-hexabromobiphenyl (^{14}C -HxBB) bound by different concentrations of individual serum proteins incubated (37°C , 60 min) in aqueous solution with 20 ng ^{14}C -HxBB.

tion: 37°C , 30 min). PBB were detected and quantified both by scintillation counting and negative chemical ionization mass spectrometry (17). The left of Figure 3 shows the distribution of PBB when they were incubated with whole blood. The vast majority of the PBB became associated with plasma (89%); 9% was associated with erythrocytes and 1% or less with each of the mononucleocytes and polymorphonucleocytes. If, however, the amount of PBB per cell is considered, there is an approximately 100-fold excess of PBB in the white cell fractions in comparison with the erythrocyte fraction. Similar conclusions were drawn from experiments using positive chemical ionization mass spectrometry on blood from exposed Michigan chemical workers (14). The relatively large amount of PBB associated with white blood cells is possibly the cause of the immunological dysfunctions that resulted from exposure to PBB.

A direct comparison between model plasma and environmentally contaminated plasma (i.e., plasma from a Michigan farmer) is shown in the midsection of Figure 3. The apolipoprotein B and A fractions were obtained by immunoprecipitation with the appropriate monospecific polyvalent antisera. The HxBB content of the separated fractions of the model plasma was determined by scintillation counting while the corresponding fractions from the Michigan sample were analyzed by negative chemical ionization mass spectrometry (17). It is seen that within experimental error the environmen-

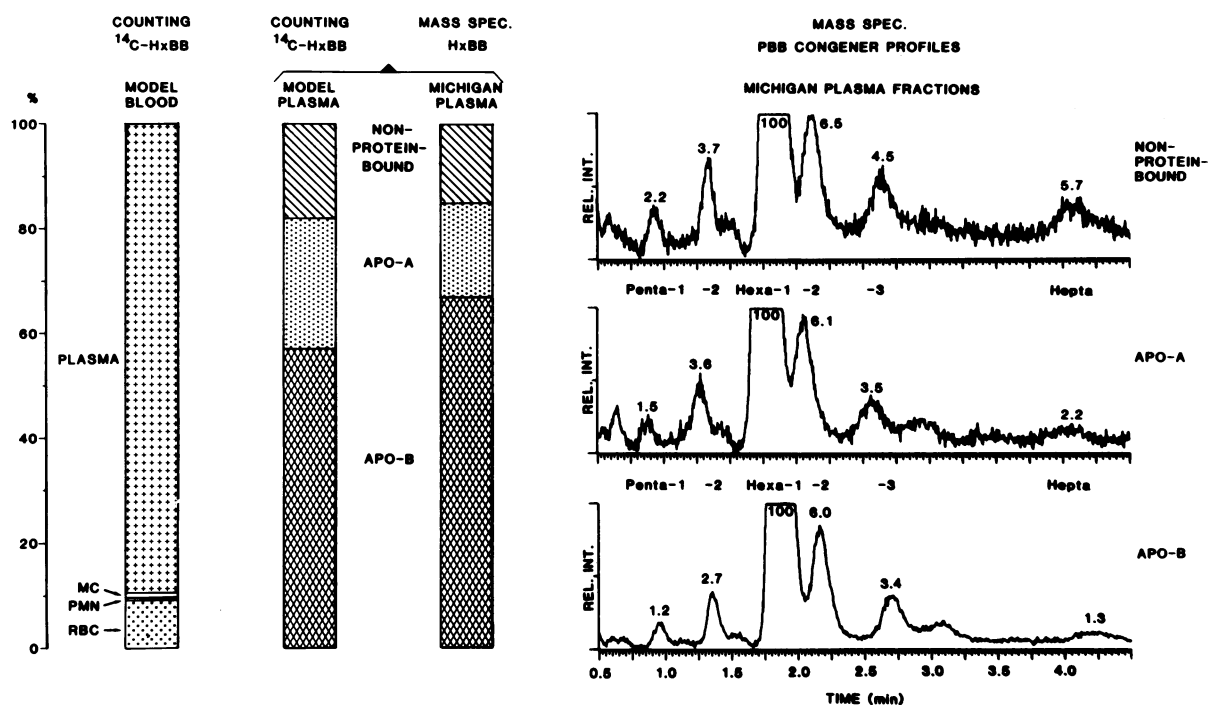


FIGURE 3. Illustration of several kinds of experiments to study the distribution and protein binding of PBB in blood. The first column shows the distribution of ^{14}C -2,2',4,4',5,5'-hexabromobiphenyl (^{14}C -HxBB) in whole blood (MC = mononucleocytes, PMN = polymorphonucleocytes, RBC = red blood cells); the second and third columns show a comparison of distribution of ^{14}C -HxBB in model plasma with that of HxBB in environmentally contaminated plasma (APO-B = apolipoprotein B, APO-A = apolipoprotein A). Selected ion monitoring profiles ($m/z = 79$) = distribution of PBB congeners in plasma fractions obtained from an environmentally contaminated blood sample. Penta-1 = 2,2',4,4',5,5'-pentabromobiphenyl; Penta-2 = 2,3',4,4',5-pentabromobiphenyl; Hexa-1 = 2,2',4,4',5,5'-hexabromobiphenyl, Hexa-2 = 2,2',3,4,4',5'-hexabromobiphenyl; Hexa-3 = 2,3',4,4',5,5'-hexabromobiphenyl; Hepta = 2,2',3,4,4',5,5'-heptabromobiphenyl.

tally contaminated samples were well represented by the *in vitro* model. The fact that the amount of HxBB not precipitated by antiapolipoprotein B could be precipitated by antiapolipoprotein A confirmed that essentially all protein-bound HxBB was bound to lipoproteins. The 3:1 ratio between the amount of PBB-bound apolipoproteins B and A reflect the 3:1 ratio of the lipid contents of these proteins rather than their amino acid content (1.6:1), and suggests that PBB binds to the lipid portions of lipoproteins and that the composition of the rest of the lipoprotein molecule is of no importance (17).

The availability of a representative model permits the *in vitro* study of the behavior of individual PBB congeners (or mixtures) at any desired absolute (or relative) concentration, which is of importance because of the limited availability of blood samples from affected Michigan residents and the very low concentrations of several PBB congeners in those samples.

Congener Profiles

In a set of experiments whole blood was spiked with equal amounts of HxBB, 2,2',4,5,5'-pentabromobiphenyl (a component of Firemaster BP-6 known to be excreted and/or metabolized) and 2,2',4,5',6-pentabromobiphenyl (not identified in Firemaster BP-6) and the PBB congeners were quantified by mass spectrometry in the cell-free, mononuclear, polymorphonuclear, and erythrocytes fractions. In spite of the huge (up to 200-fold) differences in the amounts of PBB present in the cell-free fraction with respect to other fractions, the relative amounts of the congeners, within experimental error, were the same for each fraction (17).

Mass spectrometric studies on preferential binding in environmentally contaminated samples are illustrated in the right side of Figure 3, which shows how the PBB congeners were distributed in the separated apolipoprotein B, A, and nonprotein-bound fractions of a serum sample from a Michigan subject (18). Qualitatively, these three profiles are similar and show very little, if any, selective binding of the congeners. The lack of selective binding indicates that the changes that have been observed in the PBB profiles of Michigan subjects (10,11,16) are the results of selective metabolism or excretion rather than selective absorption.

High Performance Liquid Chromatography (HPLC)

The recent availability of gel permeation columns for the separation of proteins offers an alternative approach to the study of the protein binding of PBB. Here the separating power of HPLC is combined with the detection and quantification of PBB in collected fraction from serum either by scintillation counting (model samples) or mass spectrometry (model or environmentally contaminated samples). To explore the feasibility of this approach, 1 mL serum was incubated with 40 ng of ^{14}C -

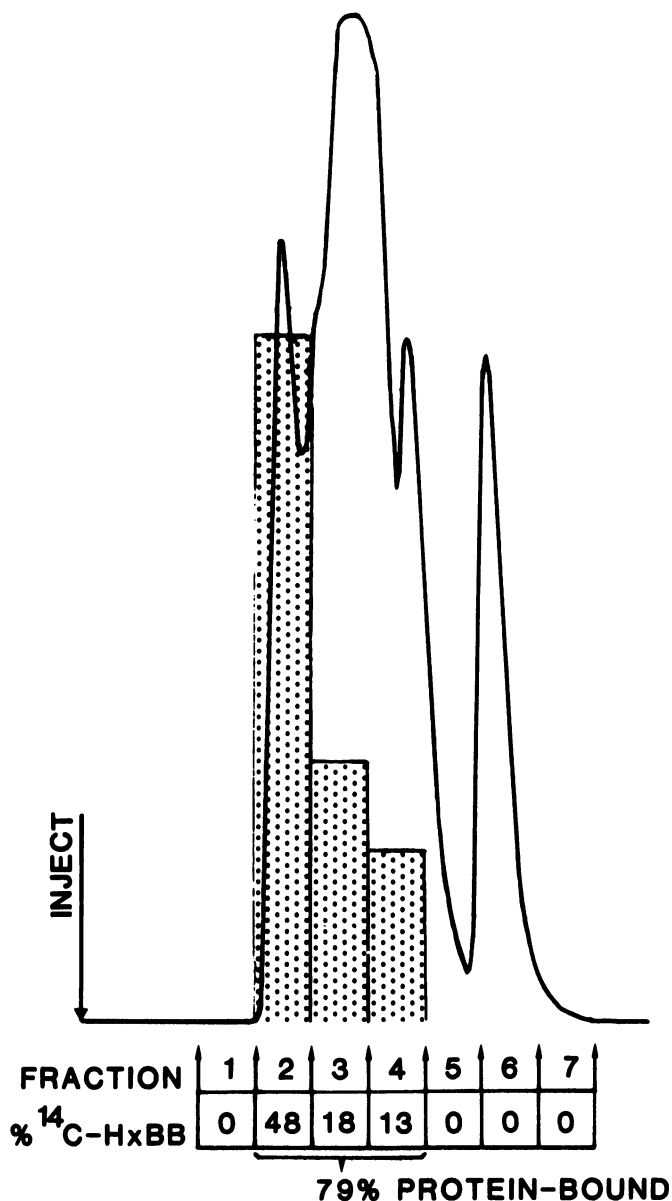


FIGURE 4. Chromatographic profile of a model plasma on a size exclusion HPLC column (Model I-250, Waters Associates). Histogram (dotted area) shows distribution of the C-2,2',4,4',5,5'-hexabromobiphenyl spike in collected fractions.

HxBB at 37°C for 1 hr. The serum was extracted with 3 mL hexane and the radioactivity of the organic phase determined. The aqueous phase was used for the HPLC experiments without any further sample preparation; 250- μL aliquots were injected directly onto the HPLC. Figure 4 shows the HPLC profile of such an aliquot on an I-250 protein column (Waters Associates, Milford, MA). Phosphate buffer (0.05 M, pH 5.7) was the eluent (flow rate = 1.0 mL/min) with detection by UV absorbance at 254 nm. Fractions were collected at 2-min intervals, and radioactivity was determined in all collected fractions. Radioactivity data are shown in Figure 4 superimposed on the HPLC profile. It is seen that about one-half of the PBB eluted in the first fraction that contained proteins (fraction 2). Based upon molec-

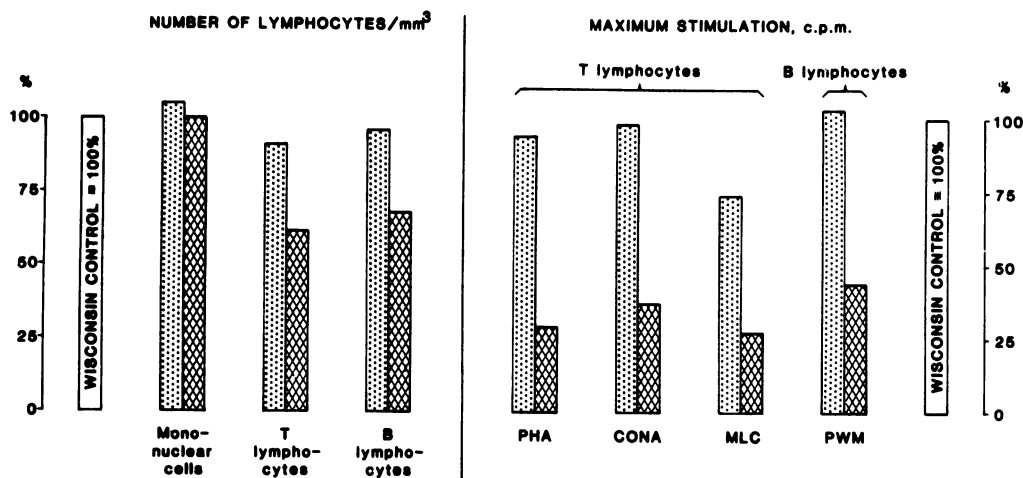


FIGURE 5. Comparison of the relative numbers and results of several immune function tests of T- and B-lymphocytes in PBB-exposed Michigan farm residents with Wisconsin farm residents ($n=46$) taken as 100%: (◻) subpopulation of Michigan subjects with normal T- and B-lymphocyte numbers and function ($n=27$); (▨) subpopulation of Michigan subjects with reduced T- and B-lymphocyte numbers and impaired immune functions ($n=18$).

ular weight calibrations it was determined that this fraction contained the apolipoprotein B (approximate MW, 3×10^6 daltons). The total radioactivity recovered from the collected protein fractions accounted for 79% of the total initial spike added. The radioactivity recovered from the hexane extract was 22%; thus the overall recovery was 101%. Improved chromatographic separation and the identification of the proteins in the collected fractions will yield additional information on the nature of the binding of PBB to serum proteins.

PBB-Induced Immune Dysfunction: Discovery in 1976

A variety of tests were used to assess the immune status of PBB-exposed Michigan dairy farmers in comparison with control populations of non-exposed Wisconsin dairy farmers and New York City residents.

The tests included total lymphocyte, and T- and B-lymphocyte counts, lymphocyte function tests using phytohemagglutinin (PHA), for T cells, concanavalin A (CONA), T cells, mixed lymphocyte culture (MLC), and pokeweed mitogen (PWM), for B cells, and determination of IgA and IgG levels. Abnormalities found in up to 40% of the Michigan population included significant decreases in the numbers of T and B lymphocytes and increases in "null" cells, and changes in the functional integrity of both T and B lymphocytes (19).

The numerical values given by Bekesi et al. (19) are shown graphically in Figure 5. The 45 Michigan farmers studied were subdivided into two populations, with 27 individuals having normal T- and B-lymphocyte numbers and functions (dotted columns) and 18 individuals with significant deviations ($p < 0.001$) from normal values (cross-hatched columns). The data for the two Michigan subpopulations are shown relative to the Wisconsin con-

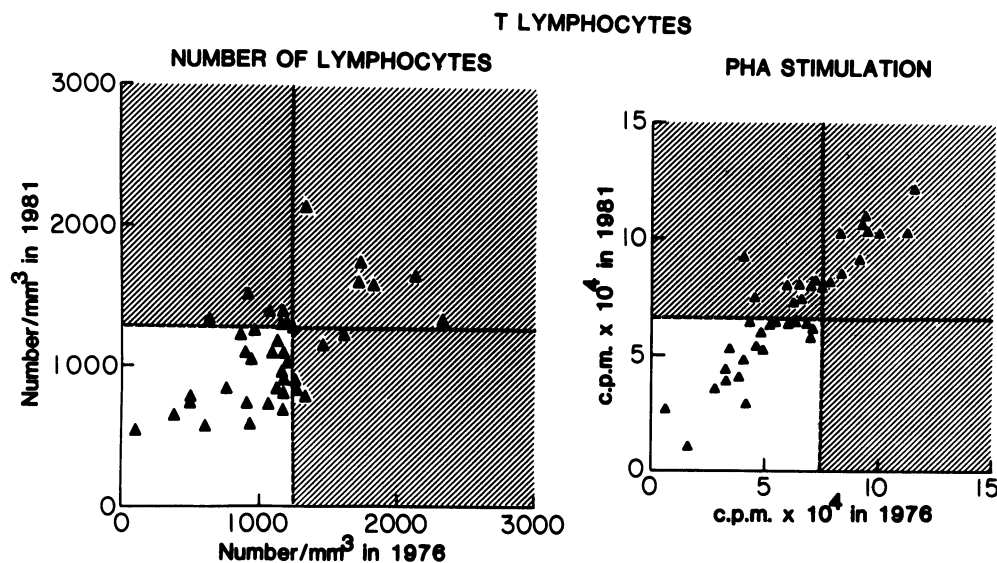


FIGURE 6. Persistence of reduced T-lymphocyte numbers and altered phytohemagglutinin response in PBB-exposed Michigan dairy farmers from 1976 to 1981. Shaded areas contain the values of the upper 95% of Wisconsin controls.

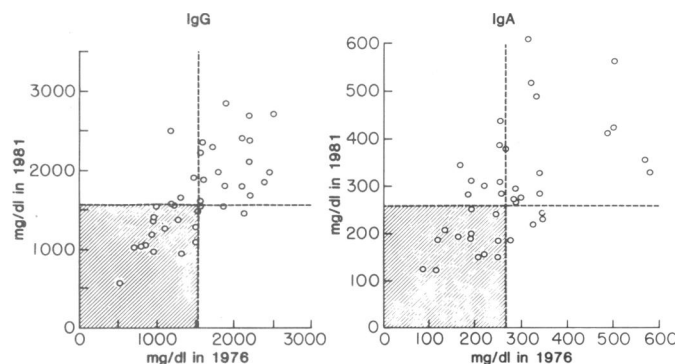


FIGURE 7. Persistence of increased IgG and IgA levels in PBB-exposed Michigan dairy farmers from 1976 to 1981. Shaded areas contain the values of the upper 95% of Wisconsin controls.

trol samples (taken as 100%). The total number of lymphocytes was apparently normal in all the Michigan residents, due to the presence of 40% "null" cells (i.e., cells having no detectable surface markers). Figure 5 also shows considerable decreases in the response to T-lymphocyte mitogens (PHA and CONA), and to a T-lymphocyte proliferation test (MLC) for the 18 affected Michigan subjects, while in the 27 nonaffected Michigan subjects the PHA and CONA values were unchanged, and the MLC values decreased only moderately with respect to Wisconsin controls. The reduction in the response to stimulation (both T- and B-lymphocytes) was approximately double the amount that would be expected on the basis of the reduced numbers of cells present in the immunodeficient individuals. Thus, in addition to the reduced numbers of T- and B-lymphocytes, the functional abilities of the remaining cells were also compromised. A likely, but not proven, reduction of the number of helper T-cells may also have contributed to the decrease in B cell functionality.

Among the immunoglobulins, increases of 83% for IgG and 43% of IgA were observed for Michigan dairy farmers with respect to Wisconsin controls.

It was concluded that as high as 40% of the Michigan residents studied had impaired immune functions in 1976.

Persistence of Immune Dysfunction: Reassessment in 1981

The immune status of 40 Michigan farm residents and 41 Wisconsin controls were re-examined in 1981. The results revealed that 17 of the Michigan subjects still suffered from an altered immune state. Figure 6 shows comparison data on the number of T-lymphocytes and on PHA stimulation obtained in 1976 and in 1981. The correlation between the two sets of tests was high; e.g., $r=0.87$ for the numbers of T-lymphocytes giving a p value of <0.01 . Scatter plots obtained for all the other cell function tests (Fig. 5) indicated a similar persistence of these manifestations of the suppressions of immunocompetence (20,21).

Increases in IgA and IgG levels encountered in 1976

were also found to be persistent (Fig. 7), with statistically significant correlations between the 1976 and 1981 data.

It is concluded that immune dysfunctions were still present in 1981, 5 years after initial testing, 8 years after initial exposure to PBB, and 3 years after the Michigan food chain was found to be clear of PBB contamination. These dysfunctions are the results of either a permanently damaged immune system or to the continuously deleterious effects of PBB available from the apparently inexhaustible store in the adipose tissues of the Michigan dairy farmers exposed to PBB a decade ago.

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